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A Comparative Cytochemical Study of the Dufour Gland in the Eusocial Bee *Apis mellifera* Linné, 1758 and *Melipona bicolor* Lepeletier, 1836

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Dufour glands of *Apis mellifera* and *Melipona bicolor* were studied under light and transmission electron microscopy, using the cytochemical techniques of mercury bromophenol blue for protein detection, imidazole-buffered osmium tetroxide selective staining of unsaturated lipids, lanthanum nitrate for intercellular junction identification and zinc-iodide-osmium tetroxide for cytoplasmic endomembrane visualization. The results in both species corroborated the lipid nature of the gland secretion and showed in *A. mellifera* the poverty of the synthetic machinery in the worker gland cells in comparison with the queen, as expected by previous biochemical analyses. The pathway of the exogenous compounds of the secretion is intracellular, since substances can penetrate the cell folds and intercellular junctions, but their access to the gland lumen is barred by the apical intercellular junctions.

Key words: Dufour gland, *Apis mellifera*, *Melipona bicolor*, cytochemistry

I. Introduction

The Dufour gland is the unique accessory gland to the bee female reproductive apparatus. In most hymenopterans, the Dufour gland consists of a sack shaped structure of epithelial cells. Its single layered epithelium is folded and produces an irregular lumen, which is lined by a thin, plain cuticle [5].

In *A. mellifera*, the Dufour gland may signal the queen fecundity condition [6], its secretion being composed of lipids [10], which indicates its pheromonal properties. Recent investigations demonstrated that the queen gland secretion attracts workers [3] and also displays worker retinue formation around the queen [12].

The secretion of the Dufour gland in workers of *A. mellifera* consists only of hydrocarbons [10], which may provoke worker alarm reaction among nestmates [3]. In queens, besides hydrocarbons, long chain wax-like esters are present [10]. According to Katzav-Gozansky *et al.* [11], only the esters are synthesized by the gland, the hydrocarbons being taken up probably from the hemolymph. In fact, the Dufour gland cells of *A. mellifera* [4] and *Bombus terrestris* [1, 2] present, in both castes, many invaginations of the basal plasmic membrane, which ramify or form loops, with many mitochondria associated on the cytosolic surface. Such structures form a cell basal labyrinth that constitutes an apparatus for absorption of exogenous substances [4–6].

Although the knowledge about this gland in eusocial bees has increased in the last decade, the exact function of the Dufour gland in such bees is far from being well understood, the majority of the investigations being carried out mainly in European bee species. To improve the knowledge of this gland in a more general panorama, a comparative cytochemical study of the Dufour gland secretory cycle in the well known *A. mellifera* and *M. bicolor*, a less studied polygynic neotropical species, was done.

II. Materials and Methods

Material

Dufour glands of workers, virgin and mated queens of *Apis mellifera*, as well as of virgin and physogastric queens of *Melipona bicolor*, captured in the apiary of the Department of Biology (UNESP – Rio Claro, Brazil), were used. At least three or more individuals of each kind were used for the gland investigation.

Methods

Mercury bromophenol blue [15]

The glands were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, during at least 2 hr. After rinsing in the same buffer, the glands were dehydrated in

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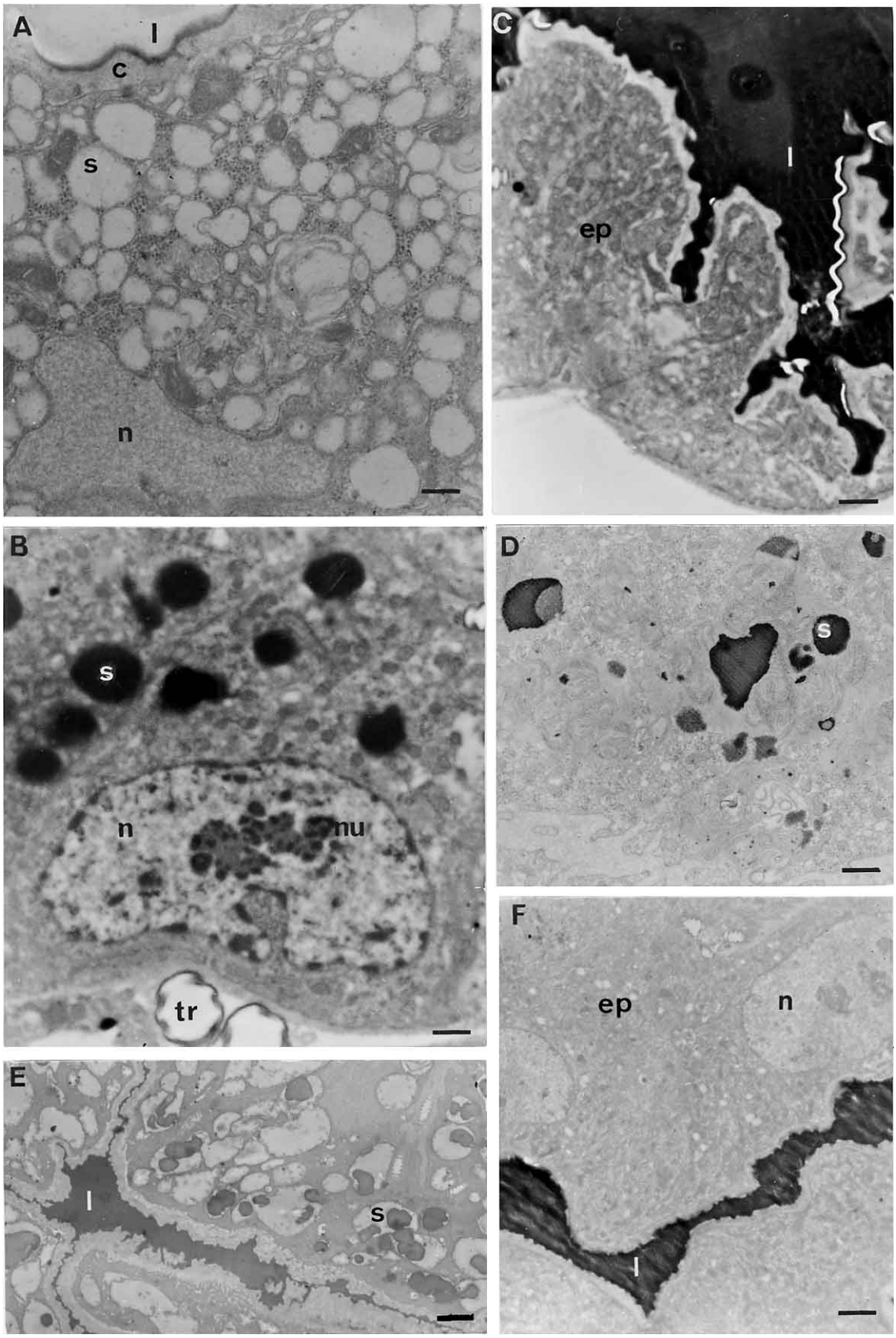

Fig. 1

an increasing alcoholic series (70 to 95%) for 10 min each. Once dehydrated, the glands were embedded and included in Leica hitoresin. The 5 μm thick sections were stained with mercury bromophenol blue (300 ml 2% acetic acid, 0.15 g bromophenol blue and 3 g $HgCl_2$), for at least 2 hr. After staining, the sections were rinsed in 0.5% acetic acid and butyric acid, for 5 min each, and observed in a Zeiss photomicroscope after being mounted in balsam.

The presence of protein is indicated by the blue color and its relative amount for differences in the blue tonality.

Imidazole-buffered osmium tetroxide [7]

The glands were fixed in 2% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodilate buffer, pH 7.4, for at least 2 hr. The gland were then rinsed twice in the same buffer, 5 min each, and then incubated in the imidazole-buffered osmium tetroxide solution (2% osmium tetroxide with 0.1 M imidazole buffer, pH 7.4), for 30 min in the darkness at room temperature. After incubation, the material was rinsed once in 0.1 M imidazole buffer and twice in PBS. The glands were dehydrated in an increasing acetone series (70 to 100%), embedded consecutively in a solution of acetone and resin in the proportion 1:1, 1:2, 1:3, for 1 hr each. The material was then embedded in resin with catalyzer (1:0.4), for 4 hr, and blockage for 24 hr at 60°C. The ultrathin sections were observed in a transmission electron microscope (Phillips), without previous contrastation. Materials prepared under routine procedure were used as control.

Lanthanum nitrate [13]

Only the glands of virgin queens of *A. mellifera* were used for this experiment.

To a solution of 2% nitrate lanthanum pH 4 was added the same volume of 2 M cacodilate buffer, pH 6.8. The mixture was used to prepare the 2.5% glutaraldehyde fixative. The glands were left in the fixative for 24 hr at room temperature. The material was then rinsed in the same buffer and prepared for routine transmission electron microscopy without post-fixation. The sections without further contrastation were observed in a transmission electron microscope (Phillips).

The lanthanum nitrate does not cross the cell membrane, but fills the open spaces [13].

Zinc iodide-osmium tetroxide [14]

Glands of forager workers, virgin and egg-laying queens of *A. mellifera* were used for the experiment.

The glands were fixed in 2% glutaraldehyde, 4% paraformaldehyde in 0.1 M sodium cacodilate buffer, pH 7.4, for at least 2 hr at room temperature. They were then rinsed twice in the same buffer plus 8% sucrose, and subsequently rinsed four times in 0.01 M Tris, pH 4.5, containing 1.13 M NaCl, 0.01 M $CaCl_2$ and 0.03 M $MgCl_2$ [16]. The fixed glands were incubated in the dark for 17 to 22 hr, at 4°C, in zinc iodide-osmium tetroxide reagent prepared as follows: 3 g zinc powder and 1 g resublimed iodine. The two solutions were then mixed and stirred for 5 min. After filtering, the solution was mixed with the Tris buffer in a 1:1 ratio. Four parts of this solution were mixed with one part 2% OsO_4 solution in distilled water for 5–10 min before use. After incubation, the glands were rinsed once in Tris buffer and twice in 0.1 M phosphate buffer, dehydrated in ethanol, and embedded in Epon/Araldite resin. The sections without further contrastation were observed in a transmission electron microscope (Phillips).

III. Results and Discussion

Proteins

The Dufour gland secretions of *A. mellifera* [10] and *M. bicolor* (data not shown) are not proteinaceous, therefore, as expected, the gland and the secretion in the lumen were not stained by the mercury bromofenol blue.

In most hymenopterans, the Dufour gland is a lipid-producing organ, the main compounds to comprising the gland secretion being esters, hydrocarbons, aldehydes, terpenes and other oxygenated non proteic substances [5, 9]. In social insects, these substances are largely used as pheromones, due to its chemical and physical properties, which make them information substances easily transmitted intraspecifically in a colony of many hundreds of individuals.

Ultrastructural localization of lipids

Imidazole, a semi-aromatic molecule, has strong affinity for metals, forming a complex with osmium tetroxide, a metal commonly used in contrast transmission electron microscopy preparations. This metal is an electron-dense substance that has an affinity for double band molecules, such as the lipids of the cellular membranes. In this way, the complex imidazole-osmium tetroxide is largely used for

Fig. 1. **A, B, C** and **D:** Transmission electron micrographs (TEM) of *M. bicolor* Dufour glands treated, except **A**, with imidazole-buffered osmium tetroxide. **A:** Dufour gland cell, showing numerous intra-cytoplasmic secretion granules (s) and an irregular nucleus (n) with chromatin decondensed. c, cuticle; l, lumen. Bar=0.4 μm. **B:** Detail of a virgin queen Dufour gland cell with the same secretion granules (s) of **A** strongly impregnated by imidazole-osmium tetroxide. n, nucleus; nu, nucleoli; tr, tracheole. Bar=0.3 μm. **C:** Strong imidazole-osmium tetroxide impregnation of the luminal (l) secretion of a virgin queen gland. ep, gland epithelium. Bar=0.5 μm. **D:** Cytoplasm detail of a physogastric queen gland cell, in which secretion granules (s) strongly impregnated by imidazole-osmium tetroxide are observed. Bar=0.5 μm. **E** and **F:** Dufour gland of *A. mellifera* treated with imidazole-osmium tetroxide. **E:** Forager worker gland, showing the cell apical portions with many intra-cytoplasmic secretion granules (s) and luminal (l) secretion impregnated by imidazole-osmium tetroxide. Bar=0.5 μm. **F:** Virgin queen gland epithelium (ep), showing strong luminal (l) impregnation by imidazole-osmium tetroxide. n, nucleus. Bar=0.6 μm.

detection of unsaturated lipids, which are the main constituents of the cellular membranes. The complex imidazole-osmium tetroxide also has affinity for lipids stored in the glandular cells of insects, as unsaturated hydrocarbons. Although imidazole is used to demonstrate the presence of lipids, the reaction is not specific and may not be considered properly as a cytochemical reaction. The treatment with imidazole and osmium tetroxide is selective for lipids, may marking other classes of substances.

The Dufour gland secretion (Fig. 1A) in *M. bicolor* queens showed strong affinity for the imidazole-osmium tetroxide (Fig. 1B, C), suggesting that its compounds present a similar nature to those found in the secretion of *A. mellifera* Dufour gland (Fig. 1F). In *M. bicolor* queens and *A. mellifera* forager workers, besides the luminal secretion (Fig. 1C, E, F), intra-cytoplasmic secretion granules were similarly contrasted (Fig. 1B, D, E).

In *A. mellifera*, where the gland is present in both castes, the worker and the queen gland secretion were impregnated by the imidazole-osmium tetroxide, the queen secretion being more intensely contrasted (Fig. 1E, F), since the queen secretion contains esters which are absent in workers. Most compounds of the worker secretion consisted of saturated hydrocarbons [10].

The gland secretory cycle is not completely known, since in both castes the cells do not present a continuous secretory activity [4]. Usually, the ultrastructural analysis reveals a great amount of luminal secretion, but hardly any trace of intra-cytoplasmatic secretory activity, as secretion vesicles or granules (Fig. 1C, F). This is due to the gland presenting markedly different phases of secretion production and release. In the secretory phase, many secretion granules are observed in the cytoplasm (Fig. 1A, B, E, D). This phase is of short duration and in *A. mellifera* the mechanism of secretion release is very curious, since the secretion is released in blocks constituted by aggregations of granules, which accumulate in the cuticle, before being released to the lumen [4]. In *A. mellifera* and *B. terrestris* the secretory phases are related with the age and fecundity condition of the bee [1, 2, 4]. The same may occur in *M. bicolor*, the physogastric queen gland cells being more active than the virgin queen gland cells (data not shown).

The present results reinforce the lipid nature of the secretion in *M. bicolor*, which was later confirmed by gas chromatography and mass spectrometry [6], as being composed of hydrocarbons in virgin queens and of hydrocarbons and esters in physogastrics.

Intercellular spaces

Lanthanum nitrate was used to investigate the types and the properties of the intercellular spaces and the permeability of the intercellular junctions to its contents. In the present case, it was used to check if compounds present in the secretion are taken up by the gland or if they transit through the intercellular spaces and reach the gland lumen without entering the cell. The lanthanum is not absorbed by the cell, but may infiltrate through the folds of the plasmic membrane and through the intercellular spaces. According to Lane [13] the septated junctions are permeable to the lanthanum.

The results showed that the lanthanum cross the basal lamina and fill up the plasmic membrane folds and intercellular spaces (Fig. 2A–C), but does not reach the subcuticular space and the gland lumen (Fig. 2C), being barred, in this case, by the sepatated junctions in the cell apical portion (Fig. 2D). Although the results showed that exogenous compounds, such as the nitrate lanthanum, are barred by the septated junctions in the cell apical gland portion, it is still possible that substances that are allowed to cross the cell membrane, such as the hydrocarbons, could be obtained from the exogenous environs. The Dufour gland presents a greatly increased exchange surface produced by the cell basal plasmic membrane invaginations (Fig. 2B), thus supporting this view. In *A. mellifera*, hydrocarbons may penetrate the Dufour gland cell by simple diffusion, without any energy expenditure. Pinocytosis, or any other form of internalization of exogenous material, was not observed.

Endomembranes

Zinc iodide-osmium tetroxide (ZIO) was introduced by Maillet [14] for observation of nervous fibers and terminations with light microscopy. Later, the technique was used in transmission electron microscopy for observation of synaptic vesicles and cellular membranes [16], and is now demonstrated to be very useful to observe endoplasmic reticulum development in insect cell investigation. The mechanism of the cytochemical reaction in not well known. Many authors attribute to the zinc iodide great affinity to the —SH groups of the intercellular membranes, while others disagree with such specificity [8].

As is well known, in workers of *A. mellifera* the Dufour gland may be considered as an excretory organ, since according to Katzav-Gozansky *et al.* [11] its cells do not produce the secretion compounds present in the lumen, which are basically hydrocarbons. In queens, the gland may be considered properly as a glandular organ, since it synthesizes the esters found in the secretion [11].

As the results showed, the workers gland synthetic machinery seems to be switched off, the vesicular smooth endoplasmic reticulum (SER) being poorly developed (Fig. 3A) in comparison with the well developed SER network of the queen gland cells (Fig. 3B–D). In workers the mitochondria were not impregnated by the ZIO, while in queens they are differently stained (Fig. 3B, D). Mitochondrial participation in the synthesis of lipids in insect cells has been recently reported [5]. This technique also revealed in queens that the SER is associated with the cell plasmic membrane, following all its contour and all the apical and basal plasmic invaginations (Fig. 3B–D), which may be related with the crossing of material absorbed from the hemolymph.

The results confirmed that the worker gland is inactive in cellular synthesis [11] and also demonstrate the specificity of the technique for the intercellular reticulum and mitochondrial membranes, since the basal lamina, the cell

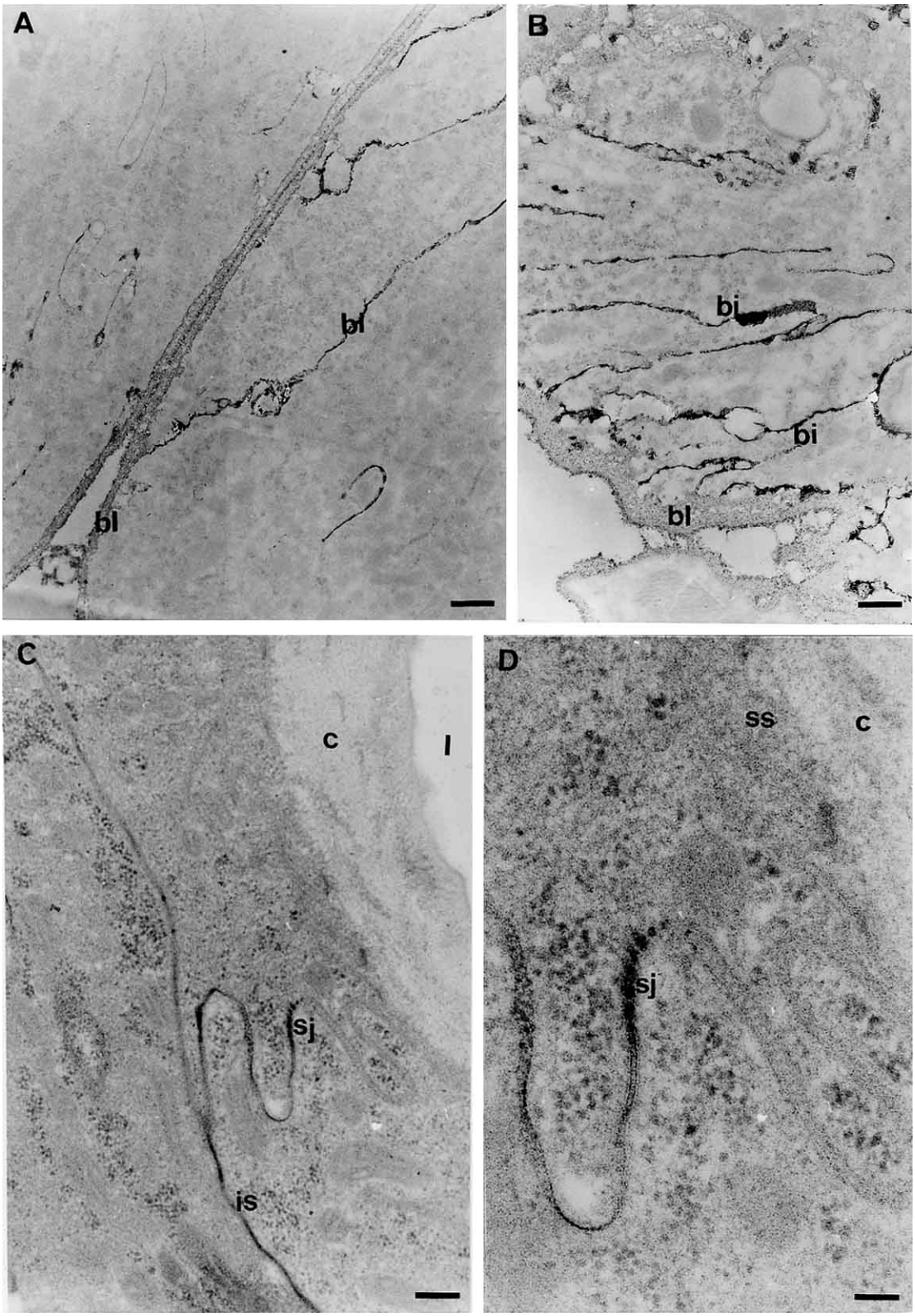

Fig. 2. TEM of virgin queen Dufour gland of *Apis mellifera* impregnated with lanthanum nitrate. **A:** Gland basal portion, showing the basal lamina (bl) and the basal plasmic membrane invaginations (bi) impregnated by the lanthanum nitrate. Bar=0.3 μm. **B:** Basal gland portion showing lanthanum impregnation in the basal lamina (bl) and in the basal plasmic invaginations (bi). Bar=0.3 μm. **C:** Apical gland portion, showing the intercellular space (is) impregnated by lanthanum nitrate, which is barred at the apical portion, being absent in the lumen (l). Bar=0.3 μm. **D:** Detail of **C**, where it is possible to notice the lanthanum nitrate being barred by the septated junctions (spj). c, cuticle; ss, subcuticular space. Bar=0.1 μm.

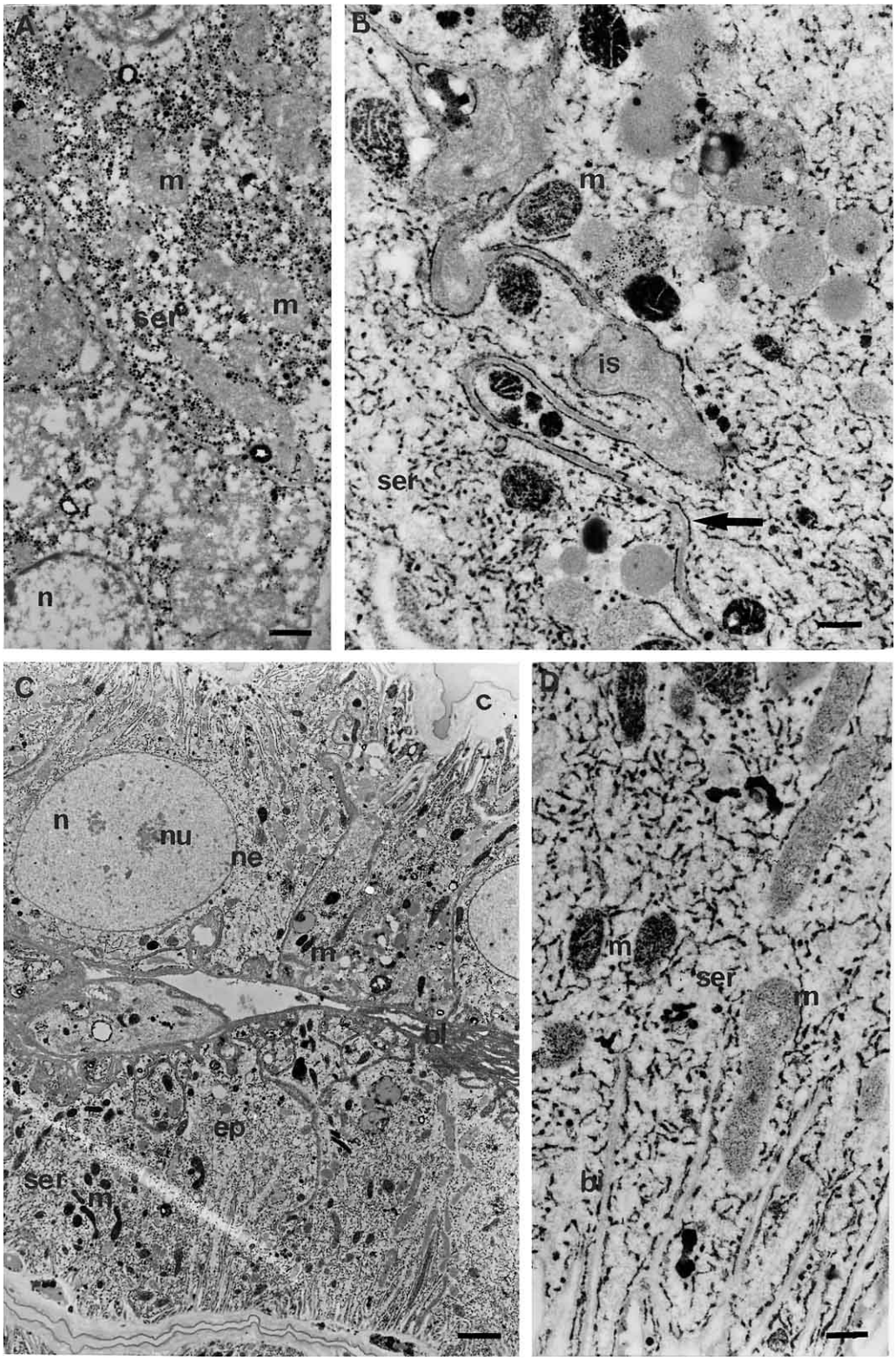

Fig. 3. TEM of *A. mellifera* Dufour glands treated with zinc iodide-osmium tetroxide (ZIO). **A:** Detail of a forager worker Dufour gland epithelium, showing the nonstained mitochondria (m) and the poorly developed vesicular smooth endoplasmic reticulum (ser). Bar=0.2 μm. **B:** Detail of virgin queen cells portion between intercellular space (is). Notice the well-developed network of tubular smooth endoplasmic reticulum (ser), which lines all the cell contours (arrow) and stain variation of mitochondria (m). **C:** Physogastric queen epithelium (ep) portion, showing the gland cells replete with smooth endoplasmic reticulum (ser) and mitochondria (m) stained by ZIO. Notice that the basal lamina (bl) and nuclear envelope (ne) are not stained. c, cuticle; n, nucleus; nu, nucleoli. Bar=3 μm. **D:** Detail of a physogastric queen gland cell basal portion, showing stain variation of mitochondria (m) and the same well developed tubular smooth endoplasmic reticulum (ser) seen in virgin queens. Bar=0.2 μm.

plasmic membrane and the nuclear envelope were not impregnated by ZIO (Fig. 3A–D).

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